ANTI-MICROBIAL TARGETING CHIMERIC PHARMACEUTICAL

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. application serial No.

5   10/077,624, filed on February 14, 2002 which is a continuation-in-part of U.S.

application serial No. 09/910,358, filed on July 19, 2001 which is a continuation-in-

part of U.S. application serial No. 09/378,577, filed on August 20, 1999 all of which

are incorporated herein by reference.

FIELD OF THE INVENTION

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This invention relates generally to the field of anti-microbial treatment and

more specifically to targeted anti-microbial treatment by chimeric constructs.

BACKGROUND OF THE INVENTION

15   The Centers for Disease Control estimates that half of more than 100 million

annual prescriptions of antibiotics are unnecessary. As a result, microbes have, in

many cases, adapted and are resistant to antibiotics due to constant exposure and

improper use of the drugs. It is estimated that the annual cost of treating drug

resistant infections in the United States is approximately $5 billion. This continued

20   emergence of anti-microbial-resistant bacteria, fungi, yeast and parasites has

encouraged efforts to develop other agents capable of killing pathogenic microbes.

Furthermore, there are urgent needs for target-specific anti-microbial agents since

many microbial pathogens reside with non-harmful commensal bacteria that are

important for the health of the human host.

25   Recent scientific studies have revealed a class of naturally occurring anti-

microbial peptides in humans, other mammals, plants, insects and other organisms. A

negative aspect of treatment with antibiotics or anti-microbial peptides is their

indiscriminate killing or inhibition of a broad spectrum of microorganisms. The

human body is home to millions of different bacteria, many of which are vital for

30   optimal health. Overuse of broad-spectrum antibiotics can seriously disrupt the

ecology of the normal human microbiota rendering humans more susceptible to

bacterial, yeast, viral, and parasitic infections. This effect is also seen with

administration of anti-microbial peptides. For example, the antibiotic peptide histatin kills most gram-positive bacteria in the oral cavity. Thus general administration of histatin can lead to undesirable effects by allowing the overgrowth of gram-negative bacteria, such as *Actinobacillus sp or Fusobacterium sp,* many of which may cause

5   periodontal diseases. Accordingly, histatin is not useful by itself for prevention of dental caries.

Another disadvantage of administration of anti-microbial peptides is their ability to damage host cells at higher concentrations since these positively charged peptides can also penetrate and disrupt eukaryotic cell membranes.

10   Previous efforts to deliver of pharmaceutically active agents to specific targets relied principally on chemically conjugating a pharmaceutically active agent to a targeting component. For example Shih et al. United States patent 5,057,313 refers to targeting delivery of drugs, toxins and chelators to specific sites in an organism by loading a therapeutic or diagnostic component onto a polymeric carrier, followed by

15   conjugation of the carrier to a targeting antibody. Hansen, United States patent 5,851,527 claims a similar invention.

A drawback to this approach is that the non-specific linkage of the pharmaceutical reagents to unknown sites on the antibody molecule used for targeting may interfere with delivery of the therapeutic agents. *See* Rodwell et al., United

20   States patent 4,671,958. Moreover, chemical modification of a targeting antibody by the nonspecific reactions during conjugation may substantively alter the antibody itself, thereby affecting its binding to targets. Furthermore, chemical linkage is very inefficient and the result is non-uniform, making the technique very difficult to use in practice.

25   More recently, there have been a number of reports of the use of recombinant techniques to produce fusion proteins for the treatment of disease. *See* Penichet and Morrison, J. Immunological Methods, 248:91-101 (2001) for review. Penichet et al. discuss efforts to treat malignant disease using a genetically engineered protein construct including an immunological component that binds specifically to tumor

30   cells and a cytokine capable of eliciting significant antitumor activity. *See, e.g. Pastan et al.* United States patent 5,981,726, and Fell, Jr. et al., United States patent 5,645,835.

However, to date there have not been any reports of directing anti-microbial agents to infected regions of humans or animals using target-specific molecules. There is a need in the art to provide methods and compositions useful for treatment of microbial organisms and microbially mediated diseases, especially microbial diseases

5   of mucosal surfaces that are not readily accessible by normal anti-microbial mechanisms provided by the immune system.

SUMMARY OF THE INVENTION

The present invention is based on the discovery that anti-microbial peptides

10   can be purposely directed to specific microbial organisms by a targeting moiety connected to the anti-microbial peptides. Accordingly the present invention provides a composition that has an anti-microbial effect on a targeted microbial organism. The present invention also provides methods of treating a microbial infection, *e.g.*, on mucosal surfaces by using the compositions provided by the present invention.

15   In one embodiment, the present invention provides a composition useful for treatment of microbial organisms. The composition comprises a targeting moiety and an anti-microbial peptide moiety, wherein the targeting moiety is coupled to the anti-microbial peptide moiety and recognizes a target microbial organism and wherein the composition has an anti-microbial effect on the target microbial organism.

20   In another embodiment, the composition comprises a targeting moiety and an anti-microbial peptide moiety, wherein the targeting moiety is a peptide, *e.g.*, polypeptide or small peptide and is fused in-frame with the anti-microbial peptide moiety. Such composition can be produced recombinantly using an expression system, *e.g.*, bacterial, yeast, or eukaryotic cell expression system, without having to

25   deal with problems associated with chemical or physical linkages.

In another embodiment, the present invention provides a method of treating a target microbial organism infection. The method comprises administering to a subject in need of such treatment an effective amount of the composition of the present invention.

30   In yet another embodiment, the present invention provides a targeting peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO. 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44,

45, 46, 47, 48, 49, 50, 51, 52, 53, 45, 55, 56, 57, 58, 59, 60, and 61.

In still another embodiment, the present invention provides a targeting peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO. 24, 25, 26, 27, 28, 29, 30, 31, 32, and 33, wherein the targeting peptide specifically binds to a microorganism of *Pseudomonas*.

In yet another embodiment, the present invention provides a targeting peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO. 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, and 51, wherein the targeting peptide specifically binds to a microorganism of *Staphylococcus*.

In another embodiment, the present invention provides a targeting peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO. 52, 53, 45, 55, 56, 57, 58, 59, 60, and 61, wherein the targeting peptide specifically binds to a microorganism of *E. coli*.

SUMMARY OF THE FIGURES

Figure 1 shows a schematic diagram of the sequential PCR reactions used to assemble the heavy chain portion of the antibody-based fusion protein.

Figure 2 shows the sequences (SEQ ID NOS: 7-13) of the primers used in the sequential PCR reactions in embodiments of the present invention.

Figure 3 shows the nucleotide sequence (SEQ ID NO: 1) encoding the anti-microbial peptide, histatin 5, the linker peptide, and the variable region of the heavy chain derived from the SWLA3 monoclonal antibody together with the amino acid sequence (SEQ ID NO: 2).

Figure 4 shows the nucleotide sequence (SEQ ID NO: 3) encoding the anti-microbial peptide, dhvar 1, the linker peptide, and the variable region of the heavy chain derived from the SWLA3 monoclonal antibody together with the amino acid sequence (SEQ ID NO: 4).

Figure 5 shows the schematic diagram of making a minibody-anti-microbial peptide fusion protein.

Figure 6 shows killing kinetics of G10 against *Pseudomonas mendocina*.

Figure 7 shows killing kinetics of G10CatC against *Pseudomonas mendocina*

Figure 8 shows killing kinetics of G10CatN against *Pseudomonas mendocina*.

Figure 9 shows killing kinetics of G10 against *Pseudomonas aeruginosa* PAK.

Figure 10 shows killing kinetics of G10CatC against *Pseudomonas aeruginosa* PAK.

Figure 11 shows long-term killing kinetics of G10 and G10CatC against *Pseudomonas mendocina*.

Figure 12 shows selective killing of *Pseudomonas mendocina* by G10CatC in mixed culture.

Figure 13 shows nonselective killing of *Pseudomonas mendocina* by G10 in mixed culture.

Figure 14 shows selective killing of PAK by G10CatC in mixed culture.

Figure 15 shows nonselective killing of PAK by G10 in mixed culture.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates in general to the targeted anti-microbial effects using a composition, *e.g.*, a chimeric construct containing a targeting moiety and an anti-microbial peptide moiety. The present invention also provides methods of treating a microbial infection using the compositions provided by the present invention.

According to the present invention, a targeting moiety can be any suitable structure that recognizes and binds to a target microbial organism. For example, a targeting moiety can be a polypeptide, peptide, small molecule, ligand, receptor, antibody, protein, or portions thereof that specifically interacts with a target microbial organism, *e.g.*, the cell surface appendages such as flagella and pili, and surface exposed proteins, lipids and polysaccharides of a target microbial organism.

In one embodiment, the targeting moiety of the present invention is a monoclonal antibody or one of various forms of a monoclonal antibody that specifically recognizes an epitope or antigen of a target microbial organism. Such epitope or antigen usually is species-specific and located on the surface of a target microbial organism. A monoclonal antibody or various forms thereof in a targeting moiety can direct an anti-microbial peptide moiety to its target site. Furthermore, it may also provide anti-microbial effect in addition to the effect provided by the anti-microbial peptide moiety since such monoclonal antibody may engage an immune

system and elicit an antibody-associated immune response, *e.g.*, humoral immune response.

A monoclonal antibody specific to a microbial organism can be made using any methods readily available to one skilled in the art. For example, as described in the U.S. Patent No. 6,231,857 (incorporated herein by reference) three monoclonal antibodies, *i.e.*, SWLA1, SWLA2, and SWLA3 have been made against *S. mutans*. Monoclonal antibodies obtained from non-human animals to be used in a targeting moiety can also be humanized by any means available in the art to decrease their immunogenicity and possibly increase their ability to elicit anti-microbial immune response of a human.

Various forms of a monoclonal antibody include, without limitation, scFv, minibody, Di-miniantibody, Tetra-miniantibody, (scFv)$_2$, Diabody, scDiabody, Triabody, Tetrabody, and Tandem diabody. A scFv usually comprises a single chain containing the variable regions of a light chain and a heavy chain, optionally joined via a linker. A minibody usually comprises the variable regions of a light chain and a heavy chain, *e.g.*, scFv joined to a heavy chain constant region, *e.g.*, about 20 amino acids or the third constant domain, C_H3 domain, either directly or via a linker, *e.g.*, about 10 to 25 amino acids. A minibody can be readily made by expressing its encoding sequence in any suitable host such as *E. coli*, yeast, or eukaryotic cell lines. A readily prepared version of a minibody usually forms a disulfide-linked dimer by virtue of the constant region, *e.g.*, C_H3 domain and a cysteine-containing linker. Various forms of a monoclonal antibodies are described in Little et al., Immunology Today, 21:364-370 (2000), which is incorporated herein by reference.

Alternatively, the targeting moiety of the present invention can include all or a portion of one or more variable regions that are capable of specifically recognizing or binding to a target microbial organism and optionally a portion of constant regions that is sufficient for dimerization. For example, the variable region of a heavy chain has three complementarity determining regions (CDRs) and is capable of binding to an antigen. One skilled in the art can readily assess the minimum variable regions required of any particular monoclonal antibody for antigen or epitope binding.

According to another embodiment of the present invention, a targeting moiety can be a targeting peptide capable of specifically binding to a microorganism, *e.g.*, a

target microbial organism. In one embodiment, the targeting peptide provided by the present invention can be identified via screening peptide libraries. For example, a phage display peptide library can be screened against a target microbial organism or a desired antigen or epitope thereof. Any peptide identified through such screening can

5   be used as a targeting peptide for the target microbial organism.

In another embodiment, the targeting peptide provided by the present invention is a peptide capable of specifically binding to *Pseudomonas*, especially *P. aeruginosa*. Such targeting peptide includes, without any limitation, a peptide containing an amino acid sequence as shown in SEQ ID NO. 24, 25, 26, 27, 28, 29,

10   30, 31, 32, or 33.

In yet another embodiment, the targeting peptide provided by the present invention is a peptide capable of specifically binding to *Staphylococcus*, especially *S. aureus*. Such targeting peptide includes, without any limitation, a peptide containing an amino acid sequence as shown in SEQ ID NO. 34, 35, 36, 37, 38, 39, 40, 41, 42,

15   43, 44, 45, 46, 47, 48, 49, 50, or 51.

In still another embodiment, the targeting peptide provided by the present invention is a peptide capable of specifically binding to *E. coli*. Such targeting peptide includes, without any limitation, a peptide containing an amino acid sequence as shown in SEQ ID NO. 52, 53, 54, 55, 56, 57, 58, 59, or 60.

20   According to the present invention, the targeting peptide of the present invention can also be a peptide obtained based on rational design. For example, one can design a targeting peptide based on the biochemical and biophysical characteristics of amino acids and the surfaces of microorganisms. In general, positively charged peptides are likely to bind negatively charged components on the

25   cell surface and vice versa. Similarly, hydrophobic peptides may bind to hydrophobic pockets on the cell surface based on hydrophobic interactions while secondary or tertiary structure of a peptide may fit into certain structures on the surface of a microorganism.

In one embodiment, the targeting peptide provided by the present invention is

30   a peptide containing an amino acid sequence as shown in SEQ ID NO. 61, 62, 63, 64, 65, 66, 67, or 68. In another embodiment, the targeting peptide provided by the present invention is a peptide containing an amino acid sequence as shown in SEQ ID

NO. 61 and capable of specifically binding to *Pseudomonas, or E. coli.*

The targeting peptides provided by the present invention can be naturally or non-naturally occurring peptides. For example, the targeting peptides provided by the present invention can be recombinantly made, chemically synthesized, or naturally

5   existing. In one embodiment, the targeting peptide contains an amino acid sequence that constitutes an internal part of a naturally occurring polypeptide. In another embodiment, the targeting peptide contains an amino acid sequence encoded by a sequence naturally existing in a genome and such amino acid sequence is not adjacent to any amino acid sequence naturally adjacent to it, *e.g.,* such amino acid sequence is

10   adjacent to a heterologous sequence in the targeting peptide.

The targeting peptide provided by the present invention can also include a peptide having an amino acid sequence that is derived or modified from a targeting amino acid sequence specifically illustrated in the present invention, provided that the derived or modified sequence still maintains or has an enhanced specificity with

15   respect to its target microbial organism. For example, the targeting amino acid sequence can be structurally modified via deletion, mutation, addition of amino acids or other structural entities, or any other structural changes as long as these changes do not alter or adversely affect the binding ability of the targeting amino acid sequence to its target microbial organism.

20   In one embodiment, the modifications to the original targeting amino acid sequence do not alter its core sequence, *e.g.,* the consensus sequence among a group of targeting amino acid sequences provided by the present invention. For example, the consensus sequence among targeting amino acid sequences of SEQ ID NO. 24, 25, 26, 27, 28, 29, 30, 31, 32, and 33 is V/Q/H-P/H-H-E-F/Y-K/H-H/A-L/H-X-X-

25   K/R-P/L (SEQ ID NO. 14), and according to the present invention any modification to a targeting amino acid sequence of SEQ ID NO. 24, 25, 26, 27, 28, 29, 30, 31, 32, and 33 should not alter the consensus sequence contained therein, *e.g.,* should not alter the V/Q/H-P/H-H-E-F/Y-K/H-H/A-L/H-X-X-K/R-P/L (SEQ ID NO. 14) sequence contained therein.

30   The targeting peptide provided by the present invention is useful for delivery of various entities to a desired locus. For example, the targeting peptide provided by the present invention can be used to deliver anti-microbial agents such as anti-

microbial peptides and detectable agents such as imaging agents, which are detectable either directly or via a secondary imaging agent. For example, the detectable agent to be delivered can be an agent which can be used with an imaging technique such as magnetic resonance imaging (MRI), positron emission tomography (PET), computer-

5   assisted tomography (CAT), X-ray, fluoroscopy and single photon emission computerized tomography.

The targeting moiety of the present invention can also be a ligand, receptor, or fragment thereof that specifically recognizes a target microbial organism. For example, the targeting moiety of the present invention can be glucan binding proteins

10   of *Streptococcus mutans* that can specifically bind insoluble glucans on the surface of *S. mutans*.

The composition of the present invention can contain one or more targeting moieties capable of targeting the same or different target microbial organisms. In one embodiment, the composition of the present invention contains one or more targeting

15   moieties capable of targeting different sites or structures of the same target microbial organism. Such composition is useful for preventing resistance of a target microbial organism to the composition.

According to the present invention, an anti-microbial peptide moiety of the composition of the present invention comprises one or more anti-microbial peptides.

20   In general, any known or later discovered anti-microbial peptides can be used for the compositions of the present invention. Anti-microbial peptides are various classes of peptides, *e.g.*, peptides originally isolated from plants as well as animals. In animals, anti-microbial peptides are usually expressed by various cells including neutrophils and epithelial cells. In mammals including human, anti-microbial peptides are usually

25   found on the surface of the tongue, trachea, and upper intestine.

Naturally occurring anti-microbial peptides are generally amphipathic molecules that contain fewer than 100 amino acids. Many of these peptides generally have a net positive charge (i.e., cationic) and most form helical structures. It is generally believed that these peptides' anti-microbial efficacy is in their ability to

30   penetrate and disrupt the microbial membranes, thereby killing the microbe or inhibiting its growth.

The anti-microbial activities of the anti-microbial peptides of the present

invention include, without limitation, antibacterial, antiviral, or antifungal activities. For example, one well-known class of anti-microbial peptides are the tachyplesins which are described as having antifungal and antibacterial activities. Andropin, apidaecin, bactencin, clavanin, dodecappeptide, defensin, and indolicidin are anti-

5   microbial peptides having antibacterial activities. Buforin, nisin and cecropin peptides have been demonstrated to have anti-microbial effects on *Escherichia. coli,*, *Shigella disenteriae*, *Salmonella typhimurium*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeroginosa*. Magainin and ranalexin peptides have been demonstrated to have anti-microbial effects on the same

10   organsims, and in addition have such effects on *Candida albicans*, *Cryptococcus neoformans*, *Candida krusei*, and *Helicobacter pylori*. Magainin has also been demonstrated to have anti-microbial effects on *herpes simplex virus*. Alexomycin peptides have been demonstrated to have anti-microbial effects on *Campylobacter jejuni*, *Moraxella catarrhalis* and *Haemophilus inflluenzae* while α defensin and β

15   pleated sheet defensin peptides have been shown to have anti-microbial effects on *Streptococcus pneumoneae*.

Histatin peptides and the derivatives thereof are another class of anti-microbial peptides, which have antifungal and antibacterial activities against a variety of organisms including *Streptococcus mutans*. MacKay, B.J. et al., Infect. Immun.

20   44:695-701 (1984); Xu, et al., J. Dent. Res. 69:239 (1990).

In one embodiment, the anti-microbial peptide moiety of the present invention contains one or more anti-microbial peptides from a class of histatin peptides and the derivatives thereof. For example, the anti-microbial peptide moiety of the present invention contains one or more derivatives of histatin including, without limitation,

25   histatin 5 having an amino acid sequence as shown in SEQ ID NO. 5 or dhvar 1 having an amino acid sequence as shown in SEQ ID NO. 6.

In another embodiment, the anti-microbial peptide moiety of the present invention contains one or more anti-microbial peptides from a class of protegrins and the derivatives thereof. For example, the anti-microbial peptide moiety of the present

30   invention contains protegrin PG-1 having an amino acid sequence RGGRLCYCRRRFCVCVGR as shown in SEQ ID NO. 15. The protegrin peptides have been shown to have anti-microbal effects on *Streptococcus mutans, Neisseria*

gonorrhoeae, *Chlamydia trachomatis* and *Haempohilus influenzae*. Protegrin peptides are described in the U.S. Patent No. 5693486, 5708145, 5804558, 5994306, and 6159936, all of which are incorporated herein by reference.

In yet another embodiment, the anti-microbial peptide moiety of the present invention contains one or more anti-microbial peptides from a class of novispirin and the derivatives thereof as described in Sawai et al., "Impact of Single –Residue Mutations on the Structure and Function of Ovispirin/Novispirin Antimicrobial . Peptides." *Protein Engineering* (in press). For example, the anti-microbial peptide moiety of the present invention contains novispirin G10 having an amino acid sequence KNLRRIIRKGIHIIKKYG as shown in SEQ ID NO. 16 for treating cariogenic organisms, *e.g., Streptococcus mutans*.

In still another embodiment, the anti-microbial peptide moiety contains one or more anti-microbial peptides including, without limitation, alexomycin, andropin, apidaecin, bacteriocin, β-pleated sheet bacteriocin, bactenecin, buforin, cathelicidin, α-helical clavanin, cecropin, dodecapeptide, defensin, β-defensin, α-defensin, gaegurin, histatin, indolicidin, magainin, nisin, protegrin, ranalexin, tachyplesin, and derivatives thereof.

The anti-microbial peptide moiety of the present invention can include one or more anti-microbial peptides, which can be the same or different anti-microbial peptides. The anti-microbial peptides of the present invention can also be modified, *e.g.,* to enhance its anti-microbial effectiveness, its cell delivery, its compatibility with the rest of the composition structure, or the manipulation of the composition in production.

The targeting moiety and the anti-microbial peptide moiety of the present invention can be coupled by various means known to one skilled in the art. For example, the targeting moiety and the anti-microbial peptide moiety can be covalently coupled or connected by a peptide linker; and the composition so formed can be constructed through molecular cloning and overexpressed and purified as one polypeptide unit in a bacterial, yeast, or eukaryotic cell expression system. Any peptide linker can be used to connect the targeting moiety and the anti-microbial peptide moiety of the present invention. In one embodiment, the peptide linker does not interfere or inhibit the activity of the targeting moiety or the anti-microbial

peptide moiety. In another embodiment, the peptide linker is from about 10 to 60 amino acids, from about 15 to 25 amino acids, or about 15 amino acids.

An anti-microbial peptide can be connected to a targeting moiety at either or both ends of the targeting moiety. In one embodiment, a targeting moiety is a peptide
5   or polypeptide which can be fused in frame at N-terminal, C-terminal, or both ends with one or more anti-microbial peptides.

In another embodiment, the targeting moiety is a targeting peptide containing an amino acid sequence as shown in SEQ ID NO. 61 and is fused in frame at either end of an anti-microbial peptide, *e.g.,* novispirin G10 as shown in SEQ ID NO. 70 and
10   71. In yet another embodiment, the targeting moiety is a targeting peptide containing an amino acid sequence as shown in SEQ ID NO. 61 and is fused in frame at the C-terminal end of an anti-micorbial peptide, *e.g.,* novispirin G10 as shown in SEQ ID NO. 70.

The composition of the present invention can be made by any suitable means known to one skilled in the art. For example, a nucleotide sequence encoding a
15   targeting moiety ligated to a nucleotide sequence encoding an anti-microbial peptide moiety, either directly or via a nucleotide sequence encoding a peptide linker, can be expressed in an appropriate expression system, *e.g.,* a commercially available bacterial, yeast, or eukaryotic cell expression system. Usually for expressing in a
20   bacterial expression system, an autocatalytic protein, *e.g.,* intein and a chitin-binding domain (CBD) are used for purification purpose. For expressing in a yeast expression system, a pheromone factor α is usually fused to the N-terminal of a coding sequence while a myocin-his tag is fused to the C-terminal of the coding sequence for easy handling of the expressed product during the purification process.

25   In one embodiment of the present invention, a commercially available yeast expression system is modified, *e.g.,* proteins used for bacterial expression systems are used for yeast expression. For example, a sequence encoding the composition of the present invention is fused with a sequence encoding pheromone factor α and a sequence encoding intein and CBD and is expressed in a yeast expression system.

30   The compositions of the present invention can be used to treat any target microbial organisms. For example, the target microbial organism of the present invention can be any bacteria, rickettsia, fungi, yeasts, protozoa, or parasites. In one

embodiment, the target microbial organism is a cariogenic organism, *e.g.,* *Streptococcus mutans.*

In another embodiment, the target microbial organisms of the present invention include, without limitation, *Escherichia. coli, Candida, Salmonella,*
5 *Staphylococcus,* and *Pseudomonas,* especially *Campylobacter jejuni, Candida albicans, Candida krusei, Chlamydia trachomatis, Clostridium difficile, Cryptococcus neoformans, Haempohilus influenzae, Helicobacter pylor, Moraxella catarrhalis, Neisseria gonorrhoeae, Pseudomonas aeroginosa, Salmonella typhimurium, Shigella disenteriae, Staphylococcus aureus,* and *Streptococcus pneumoniae.*

10 According to another feature of the present invention, the compositions of the present invention provide anti-microbial effect to target microbial organisms and can be used to treat a target microbial organism infection. An anti-microbial effect includes inhibiting the growth or killing of the target microbial organisms, or interfering with any biological functions of the target microbial organisms.

15 In general, the compositions of the present invention can be used to treat a target microbial organism infection at any place in a host, *e.g.,* at any tissue including surfaces of any implant. In one embodiment, the compositions of the present invention are used to treat a target microbial organism infection on a mucosal surface or a surface containing a biofilm. A mucosal surface usually harbors a broad
20 spectrum of microbial organisms, *e.g.,* existing as a biofilm and prefers a treatment that is least disturbing to the balance of the entire microbial organism population, *e.g.,* specific to pathogenic microbial organisms and has minimum effect on the non-pathogenic microbial population.

For example, in human mouth there usually exist many different microbes
25 including yeasts and bacteria. Most of the bacteria are non-harmful commensal bacteria that are essential for maintaining a healthy and normal microbial flora to prevent the invasion and establishment of other pathogenic microbial organisms, *e.g.,* yeast infection. Administering the composition of the present invention targets specifically to cariogenic organisms, *e.g. Streptococcus mutans* and will have
30 minimum effect on non-targeted microbial organisms, thus will not have an undesirable effect by non-targeted microbial organisms.

Many places in an animal or human body have mucosal surfaces colonized by

multiple species microbial biofilms and can be treated with the compositions of the present invention to provide targeted anti-microbial effect. For example, mouth, vagina, gastrointestinal (GI) tract, esophageal tract, respiratory tract, implants, all of which can have microbial organism infection on its mucosal surfaces.

5   In particular, *S. mutans* infection is commonly found in mouth and causes dental caries. *Porphyromonas gingivalis,* various Actinomyces species, Veillonella, spirochetes, and black-pigmented bacteroides are commonly associated with infections of gingival and surrounding connective tissues, which cause periodontal diseases. *Streptococcus pneumoniae,* nontypeable *Haemophilius influenza,* or

10   *Moraxella cararrhalis* infection is commonly found in acute otitis media (AOM) and otitis media effusion (OME) as complications of upper respiratory infections in young children.

       Helicobacter pylori (*H. pylori*) bacteria are found in the gastric mucous layer or adherent to the epithelial lining of the stomach, and cause more than 90% of

15   duodenal ulcers and up to 80% of gastric ulcers. Other GI tract infections include, without limitation, campylobacter bacterial infection, primarily *Campylobacter jejuni* associated with diarrhea, cholera caused by *Vibrio cholerae* serogroups, salmonellosis caused by bacteria salmonella such as *S. Typhimurium* and *S. enteritidis,* shigellosis caused by bacteria Shigella, *e.g., Shigella dysenteriae* and traveler's diarrhea caused

20   by enterotoxigenic *Escherichia coli* (ETEC). *Clostridium difficile* infection is also commonly found in gastrointestinal tract or esophageal tract.

       Pseudomonas organisms have been associated with common-source nosocomial outbreaks; in addition, they have been incriminated in bacteremia, endocarditis, and osteomyelitis in narcotic addicts. Infections with *Pseudomonas*

25   organisms can also occur in the ear, lung, skin, or urinary tract of patients, often after the primary pathogen has been eradicated by antibiotics. Serious infections are almost invariably associated with damage to local tissue or with diminished host resistance. Patients compromised by cystic fibrosis and those with neutropenia appear at particular risk to severe infection with *P. aeruginosa.* Premature infants; children

30   with congenital anomalies and patients with leukemia; patients with burns; and geriatric patients with debilitating diseases are likely to develop *Pseudomonas* infections. The organism is prevalent in urine receptacles and on catheters, and on the

hands of hospital staff.

The staphylococci, of which *Staphylococcus aureus* is the most important human pathogen, are hardy, gram-positive bacteria that colonize the skin of most human beings. If the skin or mucous membranes are disrupted by surgery or trauma,

5 staphylococci may gain access to and proliferate in the underlying tissues, giving rise to a typically localized, superficial abscess. Although these cutaneous infections are most commonly harmless, the multiplying organisms may invade the lymphatics and the blood, leading to the potentially serious complications of staphylococcal bacteremia.

10 These complications include septic shock and serious metastatic infections, including endocarditis, arthritis, osteomyelitis, pneumonia, and abscesses in virtually any organ. Certain strains of *S. aureus* produce toxins that cause skin rashes or that mediate multisystem dysfunction, as in toxic shock syndrome. Coagulase-negative staphylococci, particularly *S. epidermidis,* are important nosocomial pathogens, with a

15 particular predilection for infecting vascular catheters and prosthetic devices. *S. saprophyticus* is a common cause of urinary tract infection.

Yeast or Candida infections (Candidiasis) typically occur either orally (Oropharyngeal Candida or OPC) or vaginally (Vulvovaginal Candida or VVC). Candidiasis is caused by a shift in the local environment that allows Candida strains

20 (most commonly *Candida albicans*) already present on skin and on mucosal surfaces such as mouth and vagina to multiply unchecked. Gonorrhea, chlamydia, syphilis, and trichomoniasis are infections in the reproductive tract, which cause sexually transmitted diseases, *e.g.,* pelvic inflammatory disease.

The compositions of the present invention can be administered to various

25 mucosal or biofilm surfaces, *e.g.,* the mucosal surfaces described above, with each composition containing a targeting moiety corresponding to one or more specific microbial organisms of the infection, *e.g.,* the microbial organisms described above.

The composition of the present invention can also be administered to various biofilm surfaces outside of a human body, *e.g.,* industrial applications. For example,

30 in food processing industry the composition of the present invention can be administered to food processing equipments or food itself to prevent infections related to food consumption, *e.g., Salmonella* in a poultry processing facility.

The compositions of the present invention useful for treating target microbial organism infection can be administered alone, in a composition with a suitable pharmaceutical carrier, or in combination with other therapeutic agents. An effective amount of the compositions to be administered can be determined on a case-by-case

5   basis. Usually the dosage required is lower than the dosage required for an anti-microbial peptide administered without being linked to a targeting moiety, e.g., 10^{-1} lower. Factors to be considered usually include age, body weight, stage of the condition, other disease conditions, duration of the treatment, and the response to the initial treatment.

10   Typically, the compositions are prepared as a topical or an injectable, either as a liquid solution or suspension. However, solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared. The composition can also be formulated into an enteric-coated tablet, gel capsule or microsphere formulation according to known methods in the art.

15   The compositions of the present invention may be administered in any way which is medically acceptable which may depend on the disease condition or injury being treated. Possible administration routes include injections, by parenteral routes such as intravascular, intravenous, intraepidural or others, as well as oral, nasal, ophthalmic, rectal, topical, or pulmonary, e.g., by inhalation. The compositions may

20   also be directly applied to tissue surfaces. Sustained release, pH dependent release, or other specific chemical or environmental condition mediated release administration is also specifically included in the invention, by such means as depot injections or erodible implants.

In one embodiment, the compositions of the present invention are used to treat

25   or prevent cariogenic organism infections, e.g., S. mutans infection associated with dental caries and are prepared as additives to food or any products having direct contact to an oral environment, especially an oral environment susceptible to dental caries. For example, to treat or prevent dental caries one or more compositions of the present invention can be formulated into a baby formula, mouthwash, lozenges, gel,

30   varnish, toothpaste, toothpicks, tooth brushes, or other tooth cleansing devices, localized delivery devices such as sustained release polymers or microcapsules, oral irrigation solutions of any kind whether mechanically delivered or as oral rinses,

pacifiers, and any food including, without limitation, chewing gums, candies, drinks, breads, cookies, and milk.

EXAMPLES

5   The following examples are intended to illustrate but not to limit the invention in any manner, shape, or form, either explicitly or implicitly. While they are typical of those that might be used, other procedures, methodologies, or techniques known to those skilled in the art may alternatively be used.

10

Example 1.   Construction and Expression of a Histatin 5 and Dhvar 1/SWLA3 Chimeric Antibody Fusion Protein With Activity Against _S. mutans._

a.   Construction of an expression vector for an antibody-based fusion protein

15   The construct that is ultimately cloned into an IgG_1 expression vector and leads to the expression of the targeted anti-microbial fusion protein was assembled according to the following method (see FIG. **1**). The construct was assembled using sequential PCR and restriction enzymes techniques. The recognition sequence of the

20   fusion protein was derived from heavy chain sequences of SWLA3, produced by hybridoma ATCC HB 12558. _See_ Shi, United States patent 6,231,857, the disclosure of which is incorporated herein by reference, and United States patent application serial numbers 09/378,577 and 09/881,823. Sequences encoding histatin 5 or dhvar1 were inserted upstream of the variable region of the heavy chain of SWLA3. The

25   amino acid sequences used for histatin 5 and dhvar 1 are listed below:

Histatin 5 (SEQ ID NO: 5) DSHAKRHHGY KRKFHEKHHS HRGY

Dhvar 1 (SEQ ID NO: 6) KRLFKELKFS LRKY.

The source signal peptide was added upstream of the histatin 5 or dhvar1, and a glycine/serine linker was added to separate the fusion protein from the variable region

30   of the heavy chain (V_H) of the antibody. See FIG. **3** for the nucleic acid and encoded amino acid sequence for the histatin 5/SWLA3 V_H and FIG. **4** for the respective dhvar 1/SWLA3 V_H sequences. Sequential PCR reactions were used to complete the

construct according to the following method (see FIG. **2** for the nucleic acid sequence of the primers used):

1. In the first PCR reaction a plasmid carrying the V_H of SWLA3 was used as the template with primer sets 986+452 (histatin 5) or 989+452 (dhvar1). This reaction replaced the signal peptide in the original gene with the linker peptide at the 5' end of the VH and inserted a restriction site at the 3' end. The products of this reaction were isolated and used as a template in the second PCR reaction.

2. Using primer sets 987+452 (histatin 5) or 990+452 (dhvar1) in the second PCR reaction added the anti-microbial peptide upstream from the linker peptide. The restriction site at the 3' end was maintained. The products from this reaction were isolated and used as the template in the third PCR reaction.

3. With primer sets 988+452 (histatin 5) or 991+452 (dhvar1) a signal peptide and restriction site were added upstream from the anti-microbial peptide. The restriction site at the 3' end was maintained. Products from the third PCR were isolated.

4. Isolated products from the third PCR reaction were then cloned into Invitrogen's PCR2.1 vector via TOPO Cloning Kit and sequenced.

5. After the sequences of the two clones were confirmed, the inserts were moved into the IgG_1 PCR expression vector (pAH 4604) as an NheI/EcoRV fragment.

6. The final expression vectors for the histatin 5 and dhvar 1 antibody fusion proteins were named pAH 5993 and pAH 5994 respectively.

PCR conditions used were:

1. Denature @ 94°C for 40 sec.
2. Anneal @ 60°C for 40 sec.
3. Extend @ 72°C for 40 sec.
4. Amplify for 30 cycles
5. Final Extension at 72°C for 10 min.

FIG. **3** shows the nucleic acid sequence encoding the histatin 5 fusion to V_H SWLA3 and encoded amino acid sequence (SEQ ID NOS: 1 and 2) and FIG. **4** which shows the nucleic acid sequence encoding the dhvar1 fusion to V_H SWLA3 and encoded amino acid sequence (SEQ ID NOS: 3 and 4). In the figures, the bold

5   sequences represent the corresponding anti-microbial peptides, the underlined sequences represent the glycine/serine linker, and the single bolded underlined base in each sequence represents a silent point mutation. In the original sequence disclosed in Shi et al. United States patent application serial number 09/881,823, the base is guanine.

10   The variable region of the light chain (V_L) from SWLA3 was cloned into a human kappa expression vector named 5940 pAG according to the method described in Shi et al. United States patent application serial number 09/881,823. Briefly,

> (i)   DNA was prepared from the expression vectors and from the plasmid containing the correct V_L. See Current
> 15   Protocols in Immunology, Section 2.12.1 (1994) for detailed information about the vectors that express the light and heavy chain constant regions.

> (ii)   The expression vector was digested with the appropriate restriction enzyme. The digests were then electrophoresed on
> 20   an agarose gel to isolate the appropriate sized fragment.

> (iii)   The plasmid containing the cloned V_L region was also digested and the appropriate DNA fragment containing the V_L region was isolated from an agarose gel.

> (iv)   The V_L region and expression vector were then mixed together, T4 DNA ligase was added and the reaction mixture
> 25   was incubated at 16°C over night.

> (v)   Competent cells were transfected with the V_L ligation mixture and the clones expressing the correct ligation sequence

were selected. Restriction mapping was used to confirm the correct structure.

b. Transfecting Eukaryotic Cells

Ten micrograms of DNA from each expression vector, pAH 5993 (histatin 5) or pAH 5994 (dhvar 1) and 5940 pAG, was linearized by BSPC1 (Stratagene, PvuI isoschizomer) digestion and 1×10^7 myeloma cells (SP2/0 or P3X63.Ag8.653) were cotransfected by electroporation. Prior to transfection the cells were washed with cold PBS, then resuspended in 0.9 ml of the same cold buffer and placed in a 0.4 cm electrode gap electroporation cuvette. 960 microF and 200V was used for electroporation. The shocked cells were then incubated on ice in IMDM medium (Gibco, NY) with 10% calf serum.

The transfected cells were plated into 96 well plates at a concentration of 10000 cells/well. Selective medium including selective drugs such as histidinol or mycophenolic acid were used to select the cells which contain expression vectors. After 12 days, the supernatants from growing clones were tested for antibody production.

c. Analyses of histatin-5 and dhvar 1/SWLA3 chimeric antibody fusion proteins

ELISA assay was used to identify transfectomas that secrete the fusion IgG antibodies. 100 μl of 5 μg/ml goat anti-human IgG was added to each well of a 96-well ELISA plate and incubated overnight. The plate was washed several times with PBS and blocked with 3% BSA. Supernatants from above growing clones were added to the plate for 2 hours at room temperature to assay for their reactivity with goat anti-human Ig antibody. Plates were then washed and anti-human kappa antibody labeled with alkaline phosphatase diluted $1:10^4$ in 1% BSA was added for 1 hour at 37° C. Plates were washed with PBS and para-nitrophenyl phosphate in diethanolamine buffer (9.6% diethanolamine, 0.24 mM $MgCl_2$, pH 9.8) was added. Color development at OD_{405} was indicative of cells producing H_2L_2.

For the supernatants that produce IgG constant regions, their reactivity with *S. mutans* was tested as described in Shi et al., Hybridoma 17:365-371 (1998). Briefly,

A. naeslundi	ATCC12104	-	-
A. viscosus	ATCC19246	-	-
Fusobacterium nucleatum	ATCC25586	-	-
Porphyromonas gingivalis	ATCC33277	-	-

The fusion proteins showed both specificity and anti-microbial efficacy against *S. mutans*. Like the monoclonal antibodies from which they are derived, the fusion proteins bind specifically to *S. mutans*. (See Table 1). They also have anti-

5   bacterial efficacy against the bacteria, but are effective at a much lower concentration than histatin 5 alone. (See Table 2).

Table 2. Recombinant Histatin5/SWLA3 fusion antibodies
targets *S. mutans* with a great sensitivity and specificity
Minimal Inhibitory Concentrations

	S. mutans	*S. sanguis*	*Host cells*
Histatin5	~10 μM	~10 μm	>50 μM
Histatin 5/SWLA3 fusion antibodies	~0.3 μM	~30 μM	>50 μM

This observation suggests that the recognition sequence is responsible for specific binding between the fusion protein and *S. mutans*, which locally enhances the

20   concentration of histatin 5 at the bacterial cell surface. At the concentration at which the fusion protein showed antibacterial efficacy, the fusion proteins showed no inhibitory effect on other bacteria or host cells (Table 2). Accordingly, these results suggest that the basic design described herein may be useful for generating antibody-based fusion proteins for treatment of other infections and infestations.

25   **Example 2.   Construction of a Chimeric Construct Containing Minibody and Anti-microbial Peptide, and Its Expression in Yeast**

a.   Construction of SWLA3 minibody-PG-1 peptide fusion protein

A minibody is a modified antibody molecule that comprises of the variable

30   regions of the heavy and light chain (V_H and V_L) covalently linked via a short linker in a head-to-tail fashion (see Figure 5). To construct the SWLA3 minibody-PG-1

anti-microbial peptide fusion, PG-1 was linked to the N-terminus of V_H via a poly serine-glycine linker peptide, $(SGGGG)_3$ (SEQ ID NO. 17). The C-terminus of V_H was connected via a short GS linker, $(GGGS)_2$ (SEQ ID NO. 18), to the N-terminus of V_L .

5   The starting material for constructing the minibody was the anti *S. mutans* monoclonal antibody, SWLA3, as described in the United States Patent No. 6, 231,857. The anti-microbial peptide was protegrin PG-1 as described in the U.S. Patent Nos. 5693486, 5708145, 5804558, 5994306, and 6159936 and Zhao et al., FEBS lett, 1994, 346 (2-3): 285-8.

10   The coding region of the protegrin PG-1 has the following sequence: 5'- CGT GGC GGT CGC CTA TGC TAC TGT CGA CGT CGC TTT TGC GTA TGC GTG GGA CGG TCT-3' (SEQ ID NO. 19). The gene was amplified and fused with the linker region then to the VH region of SWLA3 in the same fashion as described in Example 1 for Histadin5 fusion to SWLA3.

15   To construct PG-1-SWLA3 minibody, the PG-1-V_H fragment was amplified. by PCR using primers PG-1F: 5'-GGGAATTCCGTGGCGGTCGCCTATGCTAC-3'(SEQ ID NO. 20), and VhR2: 5'-AGAGCCGCCACCCGAACCTCCGCCTGAAGAGACGGTGACTGAGG-3' (SEQ ID NO. 21). The V_L fragment was also amplified by PCR using primers V_LF2: 5'-

20 GGTTCGGGTGGCGGCTCTGATGTTGTGATGACCCAGACT-3' (SEQ ID NO. 22), and V_LR2: 5'-GGTCTAGATTCCCAGAACCCCCACCCTTACGTTTCAGCTCCAGCTTG-3' (SEQ ID NO. 23). The PG-1-V_H and V_L fragments were then joined by a second PCR utilizing the complementarities between the 3' portion of the PG-1-V_H fragment and

25 the 5' portion of the V_L fragment. The joined fragment was amplified by PCR using primers PG-1F and V_LR2. The fragment was subsequently cloned into a cloning vector, pTOPO, and the correct sequence confirmed by DNA sequencing.

b.   Expression of PG-1-SWLA3 minibody fusion in the yeast *Pichia pastoris.*

30   Yeast *Pichia pastoris* is a commercially available system for over expression of a large number of proteins including antibodies and minibodies. To overexpress the PG-1-SWLA3 minibody fusion protein in Pichia, the PG-1-V_H-V_L fragment was

cloned into the expression vector, pPICZαA at the *Eco* RI-*Xba* I sites. The cloning resulted in fusion of the N-terminus of PG-1 with the C-terminus of the α-factor signal sequence in the vector, thus allowing secretion of the fusion protein to the outside medium. The cloning also resulted in the fusion of the C-terminus of V_L to

5 the N-terminus of the *c-myc* epitope and the His-tag in the vector, allowing detection and purification of the secreted protein. The construct was amplified in *E. coli*, confirmed by sequencing, and transferred into *Pichia*. The correct expression of the fusion protein was confirmed by Western blot analysis, as well as by its ability to bind specifically to *S. mutans*, the target bacteria of the original antibody SWLA3.

10

Example 3.   Construction of Chimeric Construct Containing Surface-Binding Peptide and Anti-microbial Peptide

In addition to antibodies, some small peptide can also bind to surface structures of microorganisms or eukaryotic cells. These peptides, which we term

15 "docking moiety", allow more flexibility for the antimicrobial peptides (the killing moiety) to insert into the cell membrane for killing. These peptides can be selected from phage display libraries that contain random peptide sequences. Phage-display libraries of 8-12 amino acids peptide are commercially available. In this experiment, we have screened these libraries for peptides capable of specifically binding to a

20 target organism, which can be bacteria, yeast, or other fungi. One or more of these peptides will then be fused to the anti-microbial peptide via a peptide linker, and expressed in an appropriate host, or chemically synthesized.

a.   Selection of species-specific binding peptides with phage display

25 We employed three phage display peptide libraries (New England Biolabs) that contain >10^9 unique random-peptide-sequence-containing phage clones. The Ph.D.-C7C library displays 7-mer peptides with disulfide linkages, while the Ph.D.-7 and Ph.D.-12 libraries contain completely randomized 7-mer and 12-mer residues, respectively. The M13 filamentous phage used for the procedure carried the random

30 insert as an N-terminal fusion to the minor coat protein pIII.

Briefly, 10^{10} pfu/ml of phage library was incubated with 10^9 bacterial cells for which targeting peptides are desired. After centrifugation, unbound phage was

removed by aspiration. The pellet, which contained the target bacteria with the bound phage, was washed several times using buffers containing mild detergent to remove loosely bound phage particles, and the tightly bound phage particles were eluted. This process is termed panning.

5   The eluted phage was amplified by infecting *E. coli* F$^+$ strains. After 3-4 rounds of panning and amplification, a phage pool was obtained, which contained clones with high binding affinity for the bacteria that it was panned against. Ten to twenty phage clones from this pool were randomly picked for DNA sequencing, from which the amino acid sequence of the peptide insert was determined. By aligning the

10   amino acid sequence of multiple clones from the same phage pool, a consensus sequence for the binding peptide was constructed. This consensus sequence represents one of the binding peptides for this particular bacterium.

To confirm the binding specificity of the consensus peptide, the peptide was chemically synthesized and conjugated to FITC, a green fluorescence dye. The

15   labeled peptide was incubated with the bacteria and analyzed by fluorescent microscopy for bacterial species-specific binding. This methodology ensured that peptides selected from phage display exhibits the same binding specificity as a free peptide independent of the M13 phage particle.

20   **a-1.**   *Generation of Pseudomonas specific binding peptides using phage display*

Phage display technology was used as described above to generate targeting peptides against *Pseudomonas*. After panning against *P. aeruginosa* strain PAK cells, significant enrichment was obtained with the PhD-12 library, compared to the other libraries, after 3 rounds of panning (2 logs higher pfu/ml). A further 4th round of

25   panning was carried out using more stringent washing conditions to select for only the tightest binding clones, and the titer of recovered bound phage for PhD-12 (4.5X10^7 pfu/ml) remained high (Table 3). Ten clones were then selected from the recovered PhD-12 library phage after rounds 3 and 4. The sequences of the 3rd round clones are shown in Table 4.

30

Table 3 – Panning of PhD-12, PhD-7 and PhD-C7C

Library	1st Round (pfu/ml)		2nd Round (pfu/ml)		3rd Round (pfu/ml)		4th Round (pfu/ml)	
	input	output	input	output	input	output	input	output
PhD-12	$1X10^{10}$	$4.X10^5$	$3X10^{10}$	$3X10^5$	$2X10^{10}$	$2X10^9$	$1X10^{10}$	$4.5X10^7$
PhD-7	$5X10^{10}$	$3X10^6$	$3X10^{10}$	$2X10^4$	$1X10^{10}$	$2X10^7$	$7X10^9$	$1X10^5$
PhD-C7C	$1X10^{10}$	$1X10^6$	$3X10^{10}$	$3X10^4$	$3X10^{10}$	$6X10^6$	$1X10^{11}$	$1X10^5$

Table 4 – Round 3 clones from PhD-12

PhD-12 clone												
12:1	V	P	H	E	F	K	H	L	Q	M	K	P	SEQ ID NO. 24
12:2	V	P	H	E	F	K	H	L	Q	M	K	P	SEQ ID NO. 25
12:3	H	H	H	K	A	L	A	P	T	V	T	G	SEQ ID NO. 26
12:4	V	P	H	E	F	H	A	H	R	G	R	L	SEQ ID NO. 27
12:5	V	P	H	E	F	K	H	L	Q	M	K	P	SEQ ID NO. 28
12:6	Q	P	H	P	H	K	V	H	S	L	P	P	SEQ ID NO. 29
12:7	V	P	H	E	F	H	A	H	R	G	R	L	SEQ ID NO. 30
12:8	V	P	H	E	F	H	A	H	R	G	R	L	SEQ ID NO. 31
12:9	H	H	L	H	Y	N	P	A	F	P	G	L	SEQ ID NO. 32
12:10	Q	P	A	P	Y	I	S	S	P	S	A	S	SEQ ID NO. 33

5   Of the 3rd round sequences recovered, clones 12:1, 12:2, and 12:5 were identical, as well as clones 12:4, 12:7, and 12:8. These clones were probably originated from two clones. These two original clones, though not identical, share sequences at the N-terminal half of the peptide. Thus, a consensus sequence could be deduced from this peptide pool.

10

a-2. Generation of specific binding peptide against Staphylococcus aureus and E. coli from phage display libraries.

Phage display technology was also used to generate peptides specific for *S.*
15 *aureus* and *E. coli* . The PhD-12 library was used for the selections. The peptide

sequences from the enriched clones are summarized in Table 5 (against S. *aureus*) and Table 6 (against *E. coli*).

5   Table 5 – Amino acid sequences of clones generated against S. *aureus*

SA5.1	V	R	L	P	L	W	L	P	S	L	N	E	SEQ ID NO. 34
SA5.3	A	N	Y	F	L	P	P	V	L	S	S	S	SEQ ID NO. 35
SA5.4	S	H	P	W	N	A	Q	R	E	L	S	V	SEQ ID NO. 36
SA5.5	S	V	S	V	G	M	R	P	M	P	R	P	SEQ ID NO. 37
SA5.6	W	T	P	L	H	P	S	T	N	R	P	P	SEQ ID NO. 38
SA5.7	S	V	S	V	G	M	K	P	S	P	R	P	SEQ ID NO. 39
SA5.8	S	V	S	V	G	M	K	P	S	P	R	P	SEQ ID NO. 40
SA5.9	S	V	P	V	G	P	Y	N	E	S	Q	P	SEQ ID NO. 41
SA5.10	W	A	P	P	L	F	R	S	S	L	F	Y	SEQ ID NO. 42
SA2.2	W	A	P	P	X	P	X	S	S	L	F	Y	SEQ ID NO. 43
SA2.4	H	H	G	W	T	H	H	W	P	P	P	P	SEQ ID NO. 44
SA2.5	S	Y	Y	S	L	P	P	I	F	H	I	P	SEQ ID NO. 45
SA2.6	H	F	Q	E/N	P	L	S	R	G	G	E	L	SEQ ID NO. 46

SA2.7	F	S	Y	S	P	T	R	A	P	L	N	M	SEQ ID NO. 47
SA2.8	S	X	P	X	X	M	K	X	S	X	X	X	SEQ ID NO. 48
SA2.9	V	S	R	H	Q	S	W	H	P	H	D	L	SEQ ID NO. 49
SA2.10	D	Y	X	Y	R	G	L	P	R	X	E	T	SEQ ID NO. 50
SA2.11	S	V	S	V	G	M	K	P	S	P	R	P	SEQ ID NO. 51

Table 6 – Amino acid sequences of clones generated against *E. coli*

DH5.1	K	H	L	Q	N	R	S	T	G	Y	E	T	SEQ ID NO. 52
DH5.2	H	I	H	S	L	S	P	S	K	T	W	P	SEQ ID NO. 53
DH5.3	T	I	T	P	T	D	A	E	M	P	F	L	SEQ ID NO. 54
DH5.4	H	L	L	E	S	G	V	L	E	R	G	M	SEQ ID NO. 55
DH5.5	H	D	R	Y	H	I	P	P	L	Q	L	H	SEQ ID NO. 56
DH5.6	V	N	T	L	Q	N	V	R	H	M	A	A	SEQ ID NO. 57
DH5.7	S	N	Y	M	K	L	R	A	V	S	P	F	SEQ ID NO. 58
DH5.8	N	L	Q	M	P	Y	A	W	R	T	E	F	SEQ ID NO. 59
DH5.9	Q	K	P	L	T	G	P	H	F	S	L	I	SEQ ID NO. 60

These results suggest that these species may have multiple targets on the cell surface.

b. <u>Rational design of targeting peptides based on biochemical/biophysical</u>
5 <u>characteristics of amino acids and bacterial surfaces</u>

In addition to phage display, potential targeting peptides can also be designed based on the biochemical and biophysical characteristics of amino acids and the bacterial cell wall. For example, positively charged peptide may bind to negatively charged components on the cell surface and vice versa. Similarly, hydrophobic
10 peptides may bind to some hydrophobic pockets on the cell surface based on hydrophobic interactions, or some peptide may form a certain secondary structure that would fit into some structures on the cell surface. By applying these principles, we designed a panel of peptides and tested their binding potential against a panel of Gram-positive and Gram-negative bacteria.

15

b-1. <u>Design of peptide</u>

Peptide Cat-1 contained all cationic residues so that it could potentially bind to negatively charged molecules on bacterial surfaces. Phob-1 was made to be largely hydrophobic and attracted to the hydrophobic cell wall constituents of Gram-positive
20 bacteria. LPTG-1 contained two "cell wall binding" repeats separated by a flexible linker. These cell wall binding repeats are specifically recognized by some streptococci and are involved in bacterial adherence to surfaces. LPSB-1 and 2 consisted of repeats of the LPS binding domains found in the sheep defensin SMAP-29. Targeting peptide α-1 was designed to be 100% α-helical, a characteristic
25 common to many characterized antimicrobial peptides and an important structural feature for insertion into bacterial membranes. Anion-1 contained all negatively charged amino acid residues while Philic-1 mainly consisted of hydrophilic amino acid residues. Peptides were synthesized by standard FastMoc chemistry.

b-1. Some representatives of designed targeting peptides

30 Cat-1   LPSB-1

KKHRKHRKHRKH   RGLRRLGRRGLRRLGR

12 aa (SEQ ID NO. 61)   16 aa (SEQ ID NO. 62)

Phob-1   LPSB-2

KPVLPVLPVLPVL   VLRIIRIAVLRIIRIA

12 aa (SEQ ID NO. 63)   16 aa (SEQ ID NO. 64)

LPTG-1   α-1

LPETGGSGGSLPETG   RAHIRRAHIRR

17 aa (SEQ ID NO. 65)   11 aa (SEQ ID NO. 66)

Anion-1   Philic-1

DEDEDDEEDDDEEE   STMCGSTMCGSTMCG

14 aa (SEQ ID NO. 67)   15 aa (SEQ ID NO. 68)

b-2. Testing binding activity of the designed targeting peptides

Presented here are some examples for testing the specificity of the designed targeting peptides. The peptides were labeled at the N-terminus with the fluorescent dye FITC, and the labeled peptides were allowed to bind to the bacteria as described previously. Fluorescent microscopy was then used to determine the binding efficiency based on the brightness of the bacterial cells after peptide binding.

Table 7 – Specificity of targeting peptides against log phase cells

Bacterial strains	LPTG-1	Cat-1	Phob-1	Dye alone
E. coli Aw405	-	+	-	-
E. coli DH5α	-	++	-	-
E. coli ER2738	-	++	-	-
E. coli W3110	+	++	-	-
E. coli Mg1655	-	+	-	-

P. aeruginosa Pak	-	++++	-	-
P. mendocina	-	++++	-	-
S. mutans	-	-	-	-
S. epidermidis	+	-	-	-
S. aureus	+	-	-	-
T. denticola	-	-	-	-
M. xanthus	-	-	-	-

Data presented in Table 7 clearly show that Cat-1 binds specifically to *Pseudomonas* and *E. coli* with a much stronger binding to the former species. In contrast, Phob-1 did not bind to any of the bacterial species that we tested. LPTG-1,

5 on the other hand, showed weak binding to the two staphylococcal species.

To further confirm the specificity of Cat-1 binding to Pak cells, competition-binding experiments were conducted. Labeled Cat-1 was mixed with increasing concentrations of unlabeled Cat-1, and fluorescent labeling of the target bacterial cells was measured by fluorescent microscopy. The results demonstrated that fluorescent

10 signal of labeled Cat-1 on PAK cells was reduced upon addition of equal molar concentration of unlabeled Cat-1, and completely abolished at 10 x molar excess unlabeled Cat-1.

c.   Construction of a species-specific antimicrobial peptide with high specificity,

15 sensitivity, and much improved killing kinetics.

With the successful design of a targeting peptide (CAT-1) that can specifically bind to *Pseudomonas* and to *E. coli* to a lesser degree, one can fuse this targeting peptide to a non-specific antimicrobial peptide, making it a species-specific killing peptide. Below is an example for constructing such a species-specific antimicrobial

20 peptide and testing its specificity and killing efficiency against its targeting bacteria. This example demonstrates that this novel technology can be used to develop targeted

antimicrobial therapy against other pathogenic bacteria, fungi, and viruses.

c-1. Design of the fusion Peptides

Novispirin G10 is a wide-spectrum antimicrobial peptide, which is active against both Gram-positive and Gram-negative bacteria. Two forms of the fusion peptides were constructed. G10CatC contained the targeting peptide Cat-1 domain fused at its C-terminus to G10 through a five-residue linker, GGSGG (SEQ ID NO. 69), to allow flexibility of the targeting and killing domains. G10CatN had an opposite configuration as G10CatC in that the killing domain G10 was at the N-terminal portion of the fusion peptide. The sequences of the two fusion peptides are shown below.

G10CatC

<u>KKHRKHRKHRKH</u>GGSGG<u>KNLRRIIRKGIHIIKKYG</u> (SEQ ID NO. 70)

Cat-1 domain   G10 domain

G10CatN

<u>KNLRRIIRKGIHIIKKY</u>GGGSGG<u>SKKHRKHRKHRKH</u> (SEQ ID NO. 71)

G10 domain   Cat-1 domain

c-2. Determination of the Minimum bactericidal concentration (MBC) of the fusion peptides

To test the antimicrobial activity of the fusion peptides against target bacteria, a panel of bacteria, both gram positive and gram negative, were tested. The results of the minimum bactericidal concentration (MBC) are summarized in Table 8.

Table 8 – MBCs of G10, G10CatC and G10CatN against a selected panel of bacteria

	MBC (μM)		
Bacteria	G10	G10C	G10N
Salmonella Lt2 (ATCC)	>22.5	11.6-23.2	>23.2
Salmonella Lt7	>22.5	>23.2	>23.2
E. coli 3132 (wt)	11.25	5.8	11.6
E. coli W3110	**1.4**	**2.81**	>11.6
E. coli W3110 ampR	2.8	2.9	>23.2

E. coli Aw405	2.8	11.6	>23.2
E. coli Mg1665 (smooth)	2.8	2.9	>23.2
P. mendocina	**1.05**	**1.4-2.4**	2.8
P. aeruginosa Pak (ATCC)	**42.18**	**4.9**	17.4
P. aeruginosa Pak	54.5	1.4-2.9	45
P. fluorescens 1088	1.4	1.45	1.45
P. fluorescens 1089	>11.25	2.9	11.6
P. tabaci	5.625	2.175	>11.6
B. cepacia	23	13	11.6
Y. entercolitica	22.5	23.2	>23.2
Myxococcus	>11.25	>11.6	>11.6
Klebsiella Kay2026	>11.25	11.6-23.2	>11.6
S.aureus	>22.5	>23.2	>23.2
S. mutans 140	**3.5-7.03**	**3.6-7.25**	ND
S. mutans 159	3.5-7.03	3.6-7.25	ND
S. sanguis	3.5-7.03	3.6-7.25	ND
S. oralis	>58	>58	ND
S. mitis	>58	>58	ND

In general, G10 and G10CatC are more active against *E. coli*, *Pseudomonas*, and mutans streptococci, and less active against *Salmonella*, *Staphylococcus*, *Yersinia*, and the non-mutans streptococci. Of particular notice is that fusion of the targeting

5 peptide to the N-terminus of G10 (G10CatC) enhanced killing activity 38-fold against Pak, but decreased killing activity 2-fold against *P. mendocina*, while the same fusion did not change the killing activity against *S. mutans*. In contrast, fusion of the targeting peptide to the C-terminus of G10 (G10CatN) significantly reduced killing activity against *E. coli*, but did not change killing activity against others. These

10 results indicate that the position of the targeting peptide relative to the killing peptide plays an important role in determining the killing activity of the fusion peptide. Note that the targeting peptide CAT-1 alone does not have any antimicrobial activity.

c-3.   Determination of Killing Kinetics

For non-specific antimicrobial peptide, the number of molecules bound to the cell surface is proportional to the peptide concentration in the solution, because binding is solely dependent on passive diffusion of the peptide through solution. However, if a targeting peptide is fused to the killing peptide, a higher concentration of peptide on the target cell surface could be achieved in a short period of time due to the high affinity, active binding of the targeting peptide to the target bacterial cells.

To show that increased killing activity of the fusion peptide is due to enhanced binding to the cell surface, time-kill assays were performed using a selected number of species, *i.e. P. aeruginosa*, and *P. mendocina*. Briefly, varying concentrations of peptide G10, G10CatC, or G10CatN was added to 10^5 of target bacterial cells and survivors were counted by plating samples after 0.5, 1, 2 and 4 minutes of peptide exposure. Results are summarized in **Figures 6-10**.

Strikingly, at 9 µM concentration, the fusion peptide G10CatC achieved 2.5-log killing after 30 seconds, while treatment with G10 alone did not show significant killing until 4 minutes (1 $\log_{10}$ reduction). Concurrently, at 3 µM concentration, G10CatC treatment reduced the number of live *P. mendocina* cells to below detectable levels within 4 min, in contrast, G10 alone did not display any killing activity at the same concentration during the same time period. These data suggest that the addition of a targeting domain significantly improves the short-term killing rate against the target bacteria compared with the killing peptide alone. In contrast to G10C, G10CatN displayed no killing at any concentration tested through 4 minutes, suggesting that the G10 peptide must be fused to the C-terminus of the targeting domain to facilitate killing activity.

Of particular notice for *P. mendocina* is that although G10CatC has a 2x higher MBC than G10 alone, it kills much quicker in the kinetic analysis. This indicates that specific binding to the target cell surface allowed local accumulation of high concentration of the peptide in a short period of time (<4 minutes), thus enhanced killing kinetics.

The killing kinetics for PAK cells is similar (**Figures 9 and 10**). Here, G10 alone did not show any killing activity even at 18 µM concentration, in contrast, G10CatC killed >90% of PAK cells within 4 min at a lower concentration (9 µM).

Since G10 alone did not show significant killing activity within 4 min, another

time-kill experiment was conducted with treatments lasting up to 2 h to get a more complete picture of killing kinetics. The results are summarized in **Figure 11**. Similar to the short-term kinetic studies described above, treatment with 3μM G10CatC killed all input *P. mendocina* cells within 30 min, in contrast, it took 2 h for

5   G10 alone to kill all input *P. mendocina* cells at the same concentration. G10CatC also showed a more pronounced enhanced killing over G10 at 9μM; no surviving *P. mendocina* cells were recovered after 2 minutes of treatment with G10CatC, but a significant number of cells were recovered even after 30 minutes of G10 exposure.

10   c-4.   Determination of killing specificity

To test if G10CatC can selectively kill its target bacteria in a mixed culture, 10^5 of *P. mendocina* and *E. coli* W3110 cells were mixed and treated with 6 μM G10 or G10CatC peptides. Samples were taken at 30 sec. 1 min, 2 min, and 4 min, and survivors were counted by plating assays. Preliminary results are summarized in

15   **Figure 12 and 13**. It is apparent that treatment with G10CatC selectively reduced the number of *P. mendocina* cells from the mixed culture, and by 4 min no live *P. mendocina* cells could be detected. In contrast, the number of live W3110 cells remained nearly unchanged during the same period of treatment. As expected, treatment with G10 alone for 4 min did not affect the survival of *P. mendocina* nor

20   W3110 cells.

To further demonstrate this effect, another mixed culture killing experiment was performed, in which PAK cells were mixed with *S. mutans* cells, and the cell mixture was treated with 9 μM G10CatC or G10 alone. As shown in **Figures 14 and 15**, G10 alone did not kill any of the cells within 4 min. In contrast, G10CatC killed

25   over 90% of PAK cells, but none of *S. mutans* cells.

Taken together, these results demonstrate that G10CatC can specifically kill the bacterial species to which it specifically binds (the target cells), but not the non-target cells, although both types of cells are equally sensitive to the killing activity of the peptide (as demonstrated in MBC assay).

30   Although the invention has been described with reference to the presently preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is

limited only by the following claims.